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1. American Journal of Pathology:
   1993 Feb, 142(2):403-412
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1

Contributions of Immunocytochemistry to Gynaecological Pathology

ROBERT J. KURMAN

Surgical pathology is in the process of evolving from a morphological discipline based solely on the analysis of structural features to a functional-morphological discipline in which the traditional assessment of structure is complemented by an analysis of biochemical features. This evolution is a direct result of the introduction of immunocytochemical techniques into the surgical pathology laboratory. Standardization of the methodology and increased availability of reagents have led to widespread application of immunocytochemistry as a new 'specific' type of special stain. Successful application of immunocytochemistry, however, requires an appreciation and understanding of both its limitations and its potential. This presentation will consider areas in gynaecological pathology where immunocytochemistry, by highlighting a different frame of reference, has contributed to our understanding of the nature of disease. Attention will also be drawn to potential pitfalls in the interpretation of the results. The specific areas that will be covered are the following:

1. Localization of tumour markers (oncofetal antigens) in malignant germ cell tumours of the ovary with particular reference to α-fetoprotein (AFP) and human chorionic gonadotrophin (hCG).
2. Localization of steroid hormones at sites of synthesis, i.e. gonadal stromal tumours of the ovary, and in target tissues, i.e. endometrial neoplasia.
3. Localization of human papillomavirus (hPV) in the cervix and its role in cervical neoplasia.

METHODOLOGY

For a detailed discussion of the various types of immunocytochemical methods the reader is directed to two reviews (Taylor, 1978; Kurman and Casey, 1980). This section will be limited to a consideration of the indirect immunoperoxidase technique, the peroxidase anti-peroxidase technique and the biotin avidin method, since these are the most commonly employed in diagnostic pathology.

Fixation

The major advantage of all of these methods is that they can be performed on formalin-fixed, paraffin-embedded tissue. This obviates the need for fresh or frozen tissue, permits retrospective studies, and results in sections with excellent cellular detail. Although a wide variety of antigens can be identified in tissue fixed in 10% buffered formalin, the best fixatives for immunocytochemistry are those with a low pH such as Bouin's (pH 1.8), presumably because cations increase at a low pH and minimize the denaturation of proteins. Mercuric chloride containing fixatives such as B-5 are suitable if the mercury is removed beforehand, but Zenker-type fixatives are generally not recommended because the potassium dichromate they contain is a strong oxidizing agent which may cause marked conformational distortion of the protein molecules.

Immunostaining Procedure

Sections cut from the paraffin blocks must first be deparaffinized in xylene, cleared and then hydrated in phosphate buffered saline. Generally, the incubation with the primary antibody should be performed overnight at 4°C. High-titre, high-affinity antibodies permit the use of low concentrations, which is the most important factor in diminishing nonspecific background staining. The appropriate concentration is determined by performing serial dilutions on tissue that is known to contain the antigen being localized. The dilution that gives the best contrast between positive cells and negative background is selected.

Indirect immunoperoxidase method. After overnight incubation with the primary antiserum (usually produced in a rabbit) the sections are incubated for 30 minutes with swine anti-rabbit immunoglobulin conjugated to peroxidase. The reaction product is then visualized by the addition of diaminobenzidine (DAB) and hydrogen peroxide.

Peroxidase anti-peroxidase (PAP) method. The PAP method depends on the addition of an excess of swine anti-rabbit immunoglobulin which links the primary rabbit antiserum to the rabbit antibody in the PAP complex. The latter is a soluble antigen-antibody complex composed of peroxidase and a rabbit antibody to peroxidase. The reaction product is visualized by the addition of diaminobenzidine (DAB) and hydrogen peroxide as in the indirect method. Because of the increased sensitivity of the PAP method lower concentrations of the primary antibody can be used. This conserves antiserum and results in lower nonspecific staining.

Biotin avidin method. The biotin avidin method developed by Hsu et al (Hsu, Raine and Fanger, 1981) is the most sensitive of the three techniques. It takes advantage of the extremely high affinity between avidin, an egg white glycoprotein, and the vitamin biotin. Biotin is covalently conjugated to peroxidase molecules, enabling peroxidase to bind avidin. This biotin-avidin-peroxidase complex is linked to the primary rabbit antibody by a biotinylated goat anti-rabbit immunoglobulin. The remainder of the procedure is identical to the indirect and PAP methods.

Controls. A number of controls must be performed in order to ensure the specificity of the procedure. These include: (1) the use of tissues known to contain the antigen and tissues known to be devoid of the antigen being tested; (2) preabsorption of the antiserum with the specific antigen in an effort to abolish its specific activity, thereby confirming its specificity; and (3) substitution of the primary antiserum with normal serum from the same species. The use of bioassays and radioimmunoassays (RIA) in order to correlate the immunocytochemical localization is extremely valuable to validate the findings.

LOCALIZATION OF ONCOFETAL ANTIGENS IN GERM CELL TUMOURS OF THE OVARY

In recent years the clinical management of patients with malignant ovarian germ cell tumours has been revolutionized by the development of sensitive immunological assays. Monitoring the course of disease by serial measurement of serum levels of AFP and hCG using radioimmunoassays (RIA) has played an important role in improving survival by detecting recurrent disease before it is clinically apparent (Javadpour et al, 1978; Talerman, Haije and Baggerman, 1978; Scardino and Skinner, 1979). Immunocytochemical localization of these tumour markers in tissue sections provides a functional correlate to the traditional morphological classification of germ cell tumours (Kurman and Scardino, 1981). In addition, it can guide the clinician in selecting the appropriate markers to monitor the course of disease based on the immunocytochemical findings. Although a number of placental, embryonic and tumour-associated antigens have been identified in this group of tumours, emphasis will be placed on the role of AFP and hCG, since these represent the only tumour markers of which systematic correlative clinical and histological studies have been performed.

Immunocytochemical Findings

Dysgerminoma. In its pure form this neoplasm is not associated with either AFP or hCG. In approximately 5 per cent of dysgerminomas, syncytiotrophoblastic giant cells, which contain hCG, are present, and elevated levels of hCG can be detected in the serum (Zaloudek, Tavassoli and Norris, 1981). The syncytiotrophoblastic giant cells should not be interpreted as representing foci of choriocarcinoma, since the biphasic pattern of cyto- and syncytiotrophoblast is not present and the behaviour of these tumours more closely resembles pure dysgerminoma than a mixed germ cell tumour containing dysgerminoma and choriocarcinoma. Since the elevated level of hCG in the serum may serve as a tumour marker the presence of these cells should be noted.

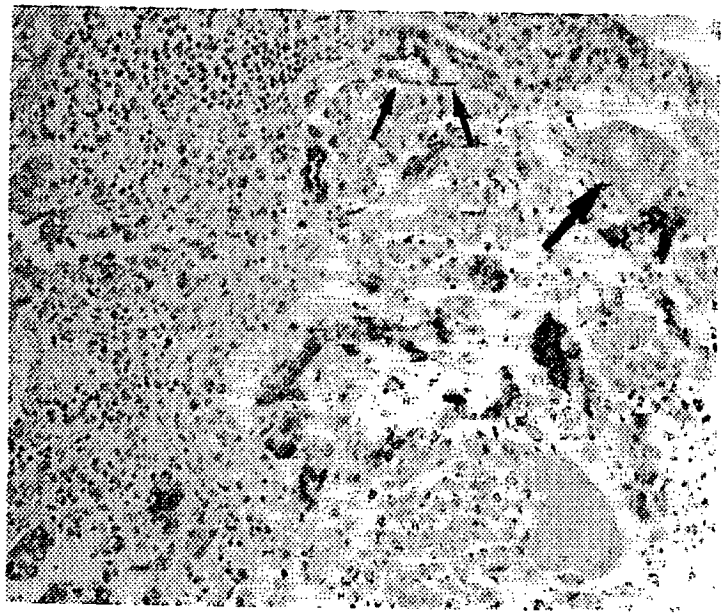


Figure 1. Embryonal carcinoma composed of masses of mononuclear embryonal carcinoma cells many of which contain AFP (black intracytoplasmic deposit). Syncytiotrophoblastic giant cell (large arrow) is negative. Note microcystic area lined by flattened cells (small arrows) some of which contain AFP (immunoperoxidase-AFP with haematoxylin counterstain).

Embryonal carcinoma. Pure embryonal carcinoma of the ovary resembles embryonal carcinoma of the adult testis but is less frequently encountered, representing approximately 5 per cent of all malignant ovarian germ cell tumours. Morphologically it is characterized by a large, primitive appearing, mononuclear cell arranged in solid masses similar to the cell that comprises the endodermal sinus (yolk sac) tumour. Syncytiotrophoblastic giant cells are also frequently present. The mononuclear cell contains AFP but not hCG (Figure 1), and the syncytiotrophoblastic giant cell contains hCG but not AFP (Kurman and Norris, 1976a) (Figure 2). Careful examination of the solid areas of embryonal carcinoma frequently discloses small foci in which the rounded mononuclear cells become spindle-shaped and arranged around slits and spaces to create a microcystic pattern (Figure 1). These microcystic areas represent the earliest morphological manifestation of yolk sac differentiation. AFP is localized in mononuclear cells within the solid masses (Figure 1) but is generally more widespread in the microcystic areas. The biochemical expression of yolk sac differentiation is the production of AFP, whereas the biochemical expression of trophoblastic differentiation is the production of hCG. The immunocytochemical findings indicate that biochemical differentiation precedes morphological differentiation. For example, localization of AFP within solid masses of cells in embryonal carcinoma indicates that

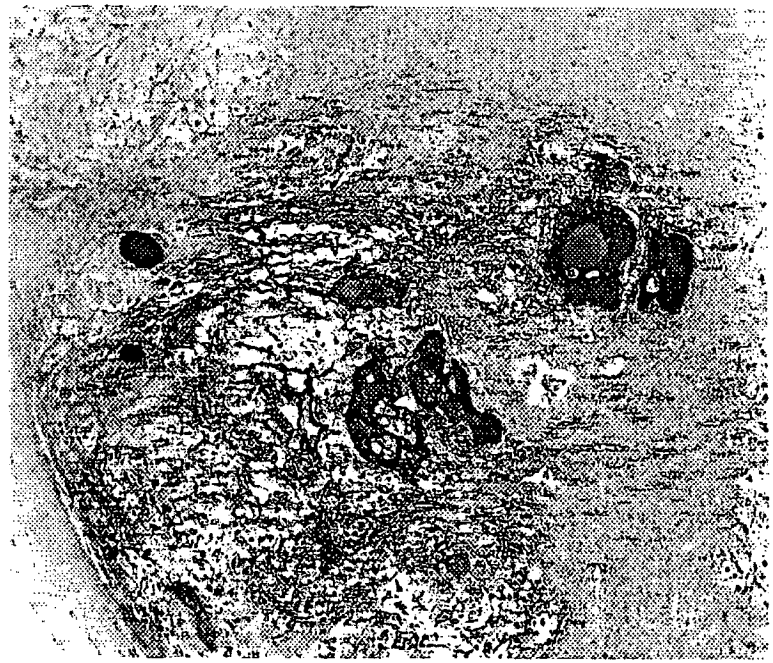


Figure 2. Embryonal carcinoma with syncytiotrophoblastic giant cells containing hCG evident by a black intracytoplasmic deposit (immunoperoxidase-hCG with haematoxylin counterstain).

biochemical differentiation towards yolk sac has occurred prior to the morphological manifestation of yolk sac differentiation, i.e. microcystic areas. Patients with embryonal carcinoma may show different patterns of marker elevations in their serum, depending on the state of biochemical differentiation of the tumour. A primitive neoplasm may fail to produce either marker, while a tumour that is biochemically differentiated along trophoblastic and yolk sac lines is associated with elevated serum levels of hCG and AFP. AFP elevation in the absence of hCG and, conversely, hCG elevation in the absence of AFP may also occur.

Endodermal sinus (yolk sac) tumour. Even in its pure form this neoplasm has a highly complex histological pattern. Although the festoon pattern containing numerous perivascular tufted structures, so-called Schiller-Duval bodies, is pathognomonic, the microcystic pattern is the most prevalent. The mononuclear cells that comprise this neoplasm contain AFP but not hCG (Kurman and Norris, 1976b) (Figure 3). The localization of AFP is often focal, and the analysis of several blocks may be required in order to identify it. Nonetheless, patients with endodermal sinus tumour have elevated serum levels of AFP which typically are higher than those found in association with embryonal carcinoma. Patients with pure endodermal sinus tumour do not have elevated levels of hCG, nor is this marker identified in tissue sections.

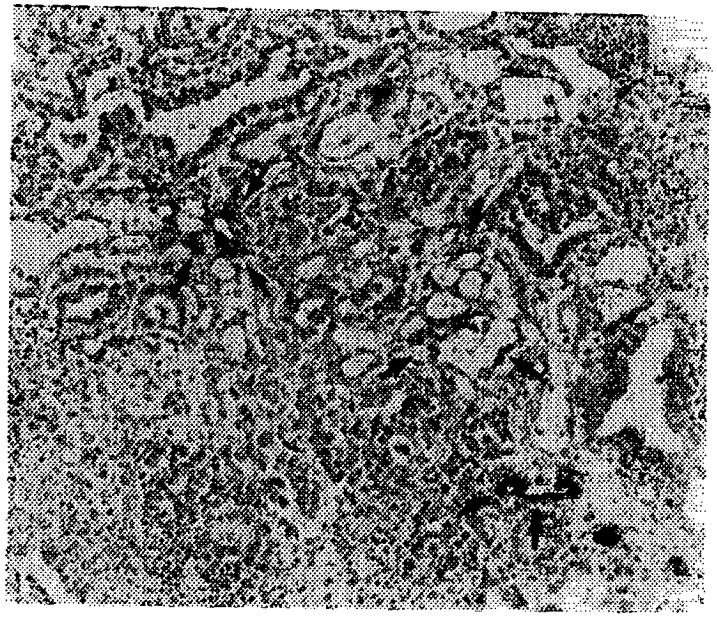


Figure 3. Endodermal sinus tumour with AFP localized in cells lining microcystic areas (arrows) (immunoperoxidase-AFP with haematoxylin counterstain).

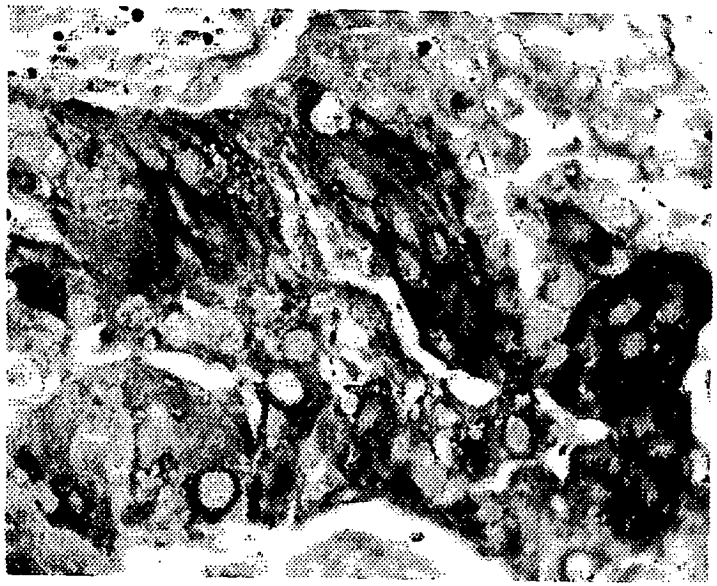


Figure 4. Choriocarcinoma with hCG localized in syncytiotrophoblast (immunoperoxidase-hCG with haematoxylin counterstain).

Choriocarcinoma. Pure nongestational choriocarcinoma of the ovary is extremely rare, representing less than 1 per cent of malignant germ cell tumours on file at the Armed Forces Institute of Pathology. More often choriocarcinoma, characterized by a biphasic pattern of cyto- and syncytiotrophoblast, is present as a component in a mixed germ cell tumour. Localization of hCG is confined to the syncytiotrophoblastic element (Figure 4); AFP is not found in choriocarcinoma (Kurman and Scardino, 1981). As in the case of gestational choriocarcinoma, patients with germ cell tumours containing choriocarcinoma have elevated serum levels of hCG.

Teratoma. Pure teratomas are typically devoid of AFP or hCG, but rarely tubular structures lined by mucinous epithelium are focally positive for AFP. Slightly elevated serum levels of AFP have been occasionally reported in patients with pure teratomas; usually neither AFP nor hCG are detected in the serum. Serum elevations of either marker should prompt a careful search for other germ cell tumour components that typically produce AFP or hCG, since this would indicate the presence of a mixed germ cell tumour. Depending on the histological composition of such a neoplasm, the prognosis and treatment may be markedly different.

Mixed germ cell tumours. These neoplasms contain two or more of any of the pure elements. The pattern of tumour marker localization in the neoplasm and the elevation in the serum reflect the histological composition of the tumour.

Limitations of Methodology

AFP. In the early immunocytochemical studies it was found that staining for AFP using fixed tissue was highly capricious and often focal despite markedly elevated serum levels of AFP (Kurman et al, 1977). Improvements in the quality of the antibodies against AFP and the development of more sensitive immunocytochemical techniques have led to more consistent results, but problems in the detection of this tumour marker in fixed tissue still exist. It appears that the denaturation occurring with routine formalin fixation causes significant alteration in the configuration of the polypeptide chains of AFP. This results in either destruction or masking of the antigenic sites and in failure of the antibody to detect them. In comparing formalin, Bouin's and Zamboni fixation, it was found that in tumours with low levels of AFP, localization of this marker by means of the peroxidase anti-peroxidase (PAP) technique occurred only in blocks fixed in Bouin's fixative (Kurman and Main, 1983, unpublished data). In addition to using Bouin's fixative, detection of AFP can be significantly improved by using more sensitive immunocytochemical methods. In a study correlating the preoperative serum level of AFP with tissue localization using the indirect peroxidase, PAP and the biotin avidin techniques, it was found that the PAP and the biotin avidin techniques were more sensitive than the indirect immunoperoxidase technique (Kurman and Main, 1983, unpublished data). In cases with low serum levels of AFP, sections stained with the indirect peroxidase technique were negative for AFP, whereas those stained with the PAP or the biotin avidin method were positive.

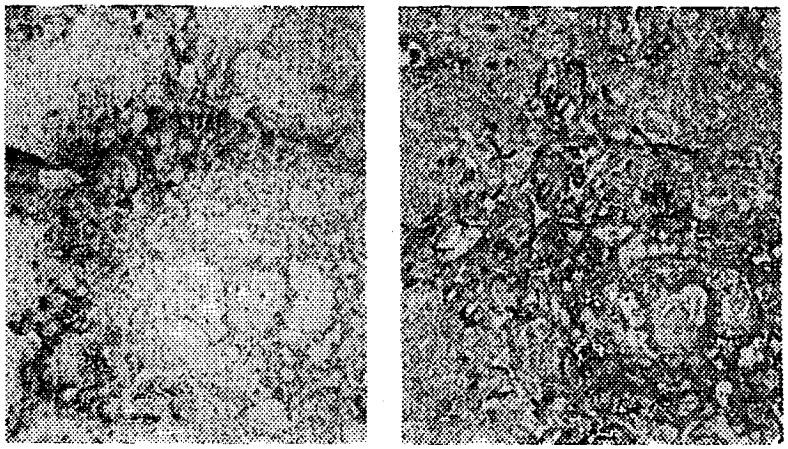
hCG. Unlike AFP, the antigenicity of hCG does not appear to be significantly impaired by routine formalin fixation. Consequently, less sensitive immunocytochemical techniques are capable of detecting even low levels of hCG. Immunoreactive hCG has been identified in tissue sections of germ cell tumours by means of the indirect immunoperoxidase technique when concomitant serum levels were barely elevated (Kurman et al, 1977). The difficulties associated with the detection of hCG in tissue sections stem from problems associated with the specificity of the antisera against hCG, not with the sensitivity of the assay.

Extensive chemical and biological homology exists between hCG and LH, but antisera generated against the beta-subunit of hCG can distinguish hCG from LH in serum using RIA. It is not well recognized, however, that these same antibodies that are effective in the RIA may show significant cross-reactivity when used in immunocytochemistry. This apparent discrepancy is due to the fact that the two immunological assays are quite different. The RIA is a competitive binding antibody assay in which a highly purified radiolabelled antigen competes with the unlabelled antigen in the test sample for binding to the specific antibody. Since only the binding of the radiolabelled tracer is measured, the quality of the antibody is less important than the purity of the labelled antigen, i.e. antibody bound to a nonspecific protein does not interfere because it is not labelled and is therefore not measured. Also the concentration of the primary antibody used in the RIA is much lower than that used in immunocytochemistry which results in a marked diminution of nonspecific binding. A 'monospecific' polyclonal antibody has a higher titre of the antibody of the desired specificity compared to the nonspecific antibodies present, and therefore dilution results in a 'wash out' of the nonspecific antibodies. In contrast, in immunocytochemistry the most important reagent is the antibody against the antigen which is being tested, since nonspecific as well as specific binding of the antibody will be visualized. Thus, if specific localization of hCG is required, a normal pituitary gland in addition to an immature placenta should be tested with the immunocytochemical method even if the antibody has been shown not to cross-react with LH using RIA.

Interpretation of Results

In addition to AFP and hCG, carcinoembryonic antigen (CEA), α1-antitrypsin (AAT), transferrin, ferritin, human placental lactogen (hPL) and pregnancy-specific β1-glycoprotein (SP1) have been identified in germ cell tumours (Kurman and Scardino, 1981). CEA, AAT, transferrin and ferritin have been localized in embryonal carcinoma cells of embryonal carcinoma and endodermal sinus tumour, CEA in mucinous epithelium in teratomas, and hPL and SP1 in syncytiotrophoblastic giant cells and in the syncytiotrophoblastic element of choriocarcinoma. Usually the various markers have a different cellular distribution, indicating that these tumours are composed of heterogeneous populations of cells that can be distinguished biochemically (Figure 5).

Correlation of the immunocytochemical and histological findings also aids in histological diagnosis by highlighting certain areas of morphological differentiation that may have been overlooked in the haematoxylin and eosin



(a) (b)

Figure 5. (a) Endodermal sinus tumour showing localization of CEA (immunoperoxidase-CEA with haematoxylin counterstain). (b) Same field as in (a) showing localization of AFP. Cells that are morphologically similar show biochemical heterogeneity (immunoperoxidase-AFP with haematoxylin counterstain).

sections. For example, the localization of AFP or hCG in an undifferentiated neoplasm of the ovary in a young woman would be strongly suggestive of a germ cell tumour. Immunocytochemical analysis also sheds light on the histogenesis of the germ cell neoplasms. The localization of AFP in embryonal carcinoma cells in solid undifferentiated masses of embryonal carcinoma and endodermal sinus tumour apart from the microcystic areas is biochemical evidence of yolk sac differentiation by both neoplasms. Similarly hCG, which reflects biochemical differentiation into trophoblast, is typically present in embryonal carcinoma as well as in choriocarcinoma. This confirms the earlier animal transplantation experiments, suggesting that embryonal carcinoma is the neoplastic progenitor of the other types of germ cell tumours apart from dysgerminoma (Stevens, 1962, 1964). Embryonal carcinoma in which no tumour markers can be identified represents the most primitive form of germ cell neoplasm.

LOCALIZATION OF STEROID HORMONES

The initial studies that utilized the PAP technique to localize oestradiol and testosterone in formalin-fixed, paraffin-embedded tissue were aimed at the detection of steroid hormones in ovarian neoplasms that synthesize steroid hormones, i.e. gonadal stromal tumours (Kurman et al, 1978; Kurman, Goebelsmann and Taylor, 1979). More recently it has been shown that the same technique and same reagents can be used to identify oestradiol bound to target tissues, i.e. breast and endometrium (Taylor et al, 1981; Farley et al,

1982). The following discussion will deal first with the role of immunocytochemistry in broadening our understanding of the cellular sites of steroid biosynthesis in granulosa-theca and Sertoli-Leydig tumours and second with the localization of oestrogen in endometrial hyperplasia and carcinoma and its relation to oestrogen receptors.

Immunocytochemical Findings

These findings are based on a study of 20 functioning granulosa-theca and Sertoli-Leydig tumours which showed clinical or biochemical evidence of steroid hormone secretion.

Granulosa-theca tumours. Granulosa cells typically contained oestradiol but also testosterone and progesterone. Luteinized theca cells also contained oestradiol and progesterone and occasionally testosterone. Although some cells that stained for one steroid hormone did not stain for another, considerable overlap occurred (Kurman, Goebelsmann and Taylor, 1979).

Sertoli-Leydig tumours. Leydig cells uniformly contained testosterone and oestradiol and occasionally progesterone. Sertoli cells were sometimes devoid of steroid hormones but often contained testosterone, oestradiol and, less commonly, progesterone. All grades of Sertoli-Leydig tumours from well

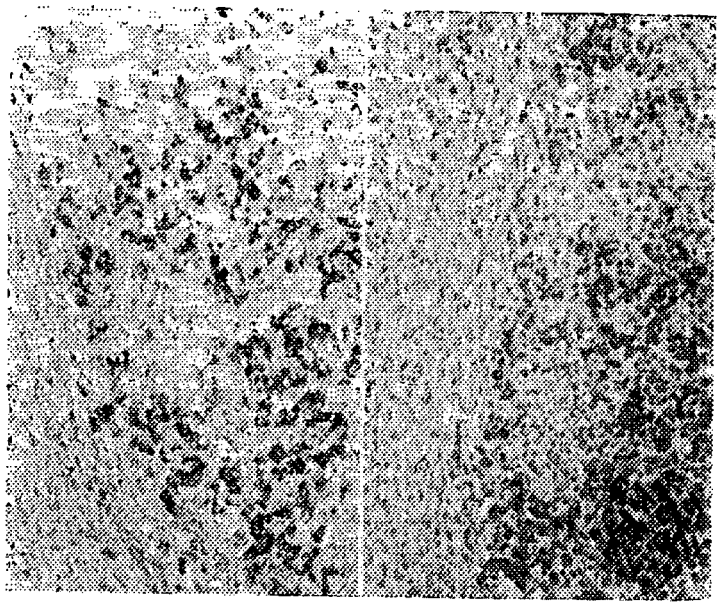


Figure 6. Poorly differentiated Sertoli-Leydig tumour. Step sections showing the same field stained for testosterone on the left (immunoperoxidase-testosterone with haematoxylin counterstain) and oestradiol on the right (immunoperoxidase-oestradiol).

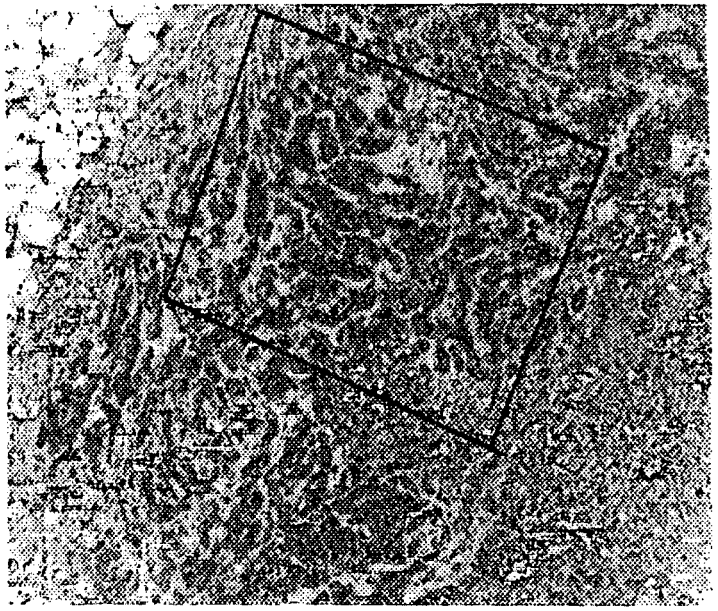


Figure 7. Metastatic poorly differentiated endometrial adenocarcinoma to the omentum showing oestrogen binding (positive cells are within bracket) (immunoperoxidase-oestradiol with haematoxylin counterstain).

differentiated to poorly differentiated showed evidence of hormone localization. Primitive spindle-shaped stromal cells found in the poorly differentiated Sertoli-Leydig tumours occasionally contained both testosterone and oestradiol (Kurman et al, 1978) (Figure 6). In contrast, lipid-laden luteinized cells observed in granulosa-theca and Sertoli-Leydig tumours did not contain any of the steroids.

Endometrial hyperplasia and carcinoma. Immunoreactive oestradiol has been localized in the glandular epithelial cells of endometrial hyperplasia and all grades of adenocarcinoma (Taylor et al, 1981; Farley et al, 1982; Kurman, Sinclair and Hartmann, unpublished data) (Figure 7). There appears to be a trend of decreasing frequency of localization of oestradiol from well to poorly differentiated adenocarcinomas, but a larger number of cases must be studied.

Limitations of Methodology

Routine processing of tissue in the surgical pathology laboratory results in loss of steroid hormones, presumably due to the use of organic solvents. Comparison of the results of PAP steroid localization using frozen and formalin-fixed, paraffin-embedded tissue revealed a similar but less intense pattern of staining in the formalin-fixed tissue, indicating that when a sensitive

technique is used, sufficient steroids remain after routine processing to permit detection (Kurman, Goebelsmann and Taylor, 1981).

Positive localization of steroid hormones in tissue sections indicates that the antiserum directed towards the steroid under study is bound to a steroid at the tissue site. The physiological basis of this localization is complex and must be considered in the interpretation of the results.

Steroid hormones are lipid soluble and therefore enter all cells. Visualization of steroids with immunocytochemical techniques is probably due to high concentrations of the steroid at a site where it is synthesized, stored or bound to a receptor. The nature of the localization of a steroid in a particular cell can only be ascertained in conjunction with other dynamic studies of hormone measurement and determination of cytosol receptor assays. The ubiquitous presence of steroids in tissue may be the reason why high background is frequently encountered with steroid immunocytochemistry. The use of the biotin avidin method helps to reduce nonspecific background staining (Kurman, Sinclair and Hartmann, 1983, unpublished data).

Finally, significant cross-reaction may occur between the antisera against testosterone and oestradiol since their chemical structure is closely related (Kurman et al, 1978). As with the protein hormones, failure to cross-react as determined by RIA is reassuring but not definitive proof of specificity. Immunocytochemistry must be performed, and the pattern of staining must be compared. Absorption of the antiserum with the specific antigen, i.e. absorption of anti-oestradiol with oestradiol in an attempt to abolish the specific staining reaction, is a useful control to test for antibody specificity.

Localization of oestradiol at target sites in the endometrium can be accomplished with formalin-fixed, paraffin-embedded tissue using the same techniques and reagents that are used for the visualization of cellular sites of hormone synthesis (Taylor et al, 1981; Farley et al, 1982). The immunocytochemical findings show a variable distribution of staining, suggesting that

Table 1. *Correlation of immunocytochemical (IC) detection of oestrogen binding with cytosol receptor assay (DCC)*[a]

Tumour	DCC[b]	IC	Concord.	Tumour	DCC[b]	IC	Concord.
Uterus	0	—	+	Breast	8	—	—
Uterus	0	—	+	Uterus	20	++	—
Uterus	0	—	+	Uterus	31	—	—
Breast	0	+	—	Uterus	41	+/—	+/—
Breast	0	—	+	Breast	52	—	—
Breast	0	+	—	Uterus	79	++	+
Breast	0	++	—	Breast	107	++	+
Breast	0	++	—	Breast	221	+++	+
Breast	0	+++	—	Uterus	271	+++	+
Uterus	4	—	+	Breast	339	++	+
Breast	6	+	+	Uterus	490	+	+

Concord. = concordance; DCC = dextran coated charcoal assay. [a] Modified and adapted from Taylor et al (1981) and Farley et al (1982). [b] Measured in fmol/mg.

there are heterogeneous populations of receptor-positive and receptor negative cells (Figure 7). Studies correlating the immunocytochemical findings with the presence of high-affinity oestrogen receptor protein, as measured by cytosol receptor assays, have shown discordant results (Table 1). Although it is conceivable that these discrepancies are due to differences in sampling it is likely that the immunocytochemical technique detects other types of oestrogen binding besides the high-affinity oestrogen receptor protein. It is noteworthy that there is concordance between the two methods when the oestrogen receptor protein level exceeds 79 fmol/mg. This suggests that the immunocytochemical assay is less sensitive than the cytosol assay and that it may be unreliable for low levels of oestrogen receptor protein. The significance and the role of the immunocytochemical assay for oestrogen binding must be determined from studies correlating long-term follow up with the immunocytochemical findings.

Interpretation of Results

Traditionally, hormone synthesis by functioning ovarian (gonadal stromal) tumours has been related to specific cell types. Oestrogen synthesis has been attributed to theca cells and testosterone synthesis to Leydig cells. Nonluteinized granulosa cells and Sertoli cells were generally considered to be inactive. Classification of these tumours in the past has been based on their endocrine manifestations or on their morphological features. Both types of classification are compromised by the fact that these neoplasms often present with variable hormonal profiles and frequently display complex arrays of interrelated histological patterns. Correlation of histological features with endocrine manifestations has frequently been unclear.

Clinical studies in which hormone measurement has been performed on the venous effluent of tumours, peripheral venous samples, tissue extracts and in vitro incubation studies using labelled precursors are in accordance with the immunocytochemical findings (Kurman, Goebelsmann and Taylor, 1981). Thus, it appears that the various cell types that comprise functioning ovarian tumours have the capacity to produce a wide range of steroid hormones. In most instances a particular hormone is the predominant product of a particular cell, but in certain circumstances the same cell type may produce a different hormonal profile. Recognition of the diverse capacity for steroid synthesis by these cells therefore explains the variable and sometimes paradoxical hormonal expression that such tumours may display.

In view of the multiple factors that may account for steroid hormone localization, it is hazardous to classify an ovarian tumour as belonging to the gonadal stromal category on the basis of the immunocytochemical findings alone.

LOCALIZATION OF HUMAN PAPILLOMAVIRUS (HPV) IN THE CERVIX

Attention is currently being focused on the role of hPV in the aetiology of cervical dysplasia and its possible role in cervical cancer (Meisels, Morin and

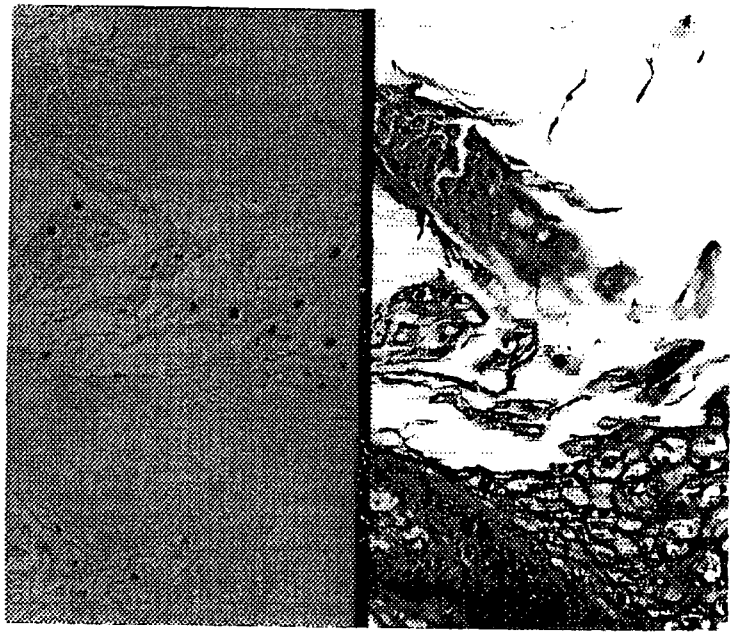


Figure 8. Condyloma acuminatum of vulva showing intranuclear localization of hPV antigens in left panel (immunoperoxidase-papillomavirus antigens, no counterstain) corresponding to koilocytotic cells in haematoxylin and eosin section in right panel.

Casa-Cordero, 1982). Evidence for hPV in cervical dysplasia has been largely based on studies showing a close cytological and histological similarity to vulvar and perianal condylomas, lesions known to be caused by PV (Meisels, Fortin and Roy, 1977; Ludwig, Lowell and LiVolsi, 1981) (Figure 8). Reports identifying viral particles by electron microscopy in cervical dysplasia compatible with papovavirus (Reid et al, 1980) have added weight to this argument, but the demonstration of PV antigens by immunocytochemistry has provided more convincing evidence supporting this view (Woodruff et al, 1980; Kurman et al, 1981, 1982; Morin et al, 1981).

In the past, attempts to study the role of PV in human neoplasms have been hampered for several reasons. First, the remarkable plurality of hPV, which is composed of at least 18 distinct serotypes (hPV-1 to hPV-18), made the screening of tissue sections for PV antigens impractical because antiserum prepared against intact virus of one type failed to cross-react with other types. Second, because of the high degree of species specificity, PV-related lesions in humans could not be induced in other species, precluding the development of an animal model. Third, the inability to propagate the virus in tissue culture made in vitro characterization of hPV impossible. Recent advances in immunocytochemistry and molecular virology have circumvented some of these problems. Thus, it has been shown that an antiserum prepared against a common (genus-specific) antigen now permits detection of all types of human

and animal PV using the PAP technique on formalin-fixed, paraffin-embedded tissue (Jenson et al, 1980). Furthermore, it was demonstrated that molecular hybridization performed under nonstringent techniques detects conserved polynucleotide sequences among the various types of PV DNAs that are not detected under stringent conditions (Law, Lancaster and Howley, 1980). Using the modified hybridization techniques, it is now possible to assay human tissues for the presence of PV-specific DNA sequences. The following summarizes the findings in 322 cases of cervical dysplasia (mild, moderate and severe) and carcinoma in situ (CIS) examined for the presence of PV structural antigens using the PAP technique on formalin-fixed, paraffin-embedded tissue (Kurman, Jenson and Lancaster, 1983). The immunocytochemical findings will be correlated with the morphological features and with the presence of PV DNA sequences.

Immunocytochemical Findings

The frequency of localization of PV antigens was found to diminish in relation to the severity of the dysplastic process. PV antigens were present in 43 per cent of mild dysplasia, 15 per cent of moderate dysplasia, 17 per cent of severe dysplasia and 10 per cent of CIS. PV antigens were frequently localized directly in mild and moderate dysplasia but were only rarely identified directly within severe dysplasia. PV antigens were never localized within areas of CIS. In the high-grade lesions (severe dysplasia and CIS) PV antigens were present within areas of mild and moderate dysplasia adjacent to the high-grade lesions. PV antigens were never identified within areas of metaplastic squamous epithelium in the transformation zone where the dysplastic lesions arose.

Morphological features associated with the presence of PV infection, i.e. koilocytosis, nuclear wrinkling, binucleation and multinucleation, dyskeratosis and epithelial spikes, were found in a higher proportion of cases. These features were found in 95 per cent of mild dysplasia, 77 per cent of moderate dysplasia, 64 per cent of severe dysplasia and 44 per cent of CIS.

Mild dysplasia. These lesions were characterized by cells in the superficial layers exhibiting varying degrees of nuclear enlargement and wrinkling (papillomavirus-induced atypia, PVA) overlying a hyperplastic zone of basal and parabasal cells (papillomavirus-associated hyperplasia, PVH). PV structural antigens were invariably intranuclear and localized in a focal or diffuse distribution in cells showing PVA in the superficial layers of the epithelium (Figure 9). Positive cells in the intermediate layers frequently displayed cytoplasmic vacuolation (koilocytosis), although vacuolation was absent close to the surface. Binucleate cells were occasionally positive, but multinucleate cells were not. Cells showing a positive reaction could not be distinguished cytologically from those that were negative.

Moderate dysplasia. In contrast to mild dysplasia these lesions showed a greater degree of proliferation of the basal and parabasal layers (PVH) involving one-half to two-thirds the thickness of the epithelium and a greater

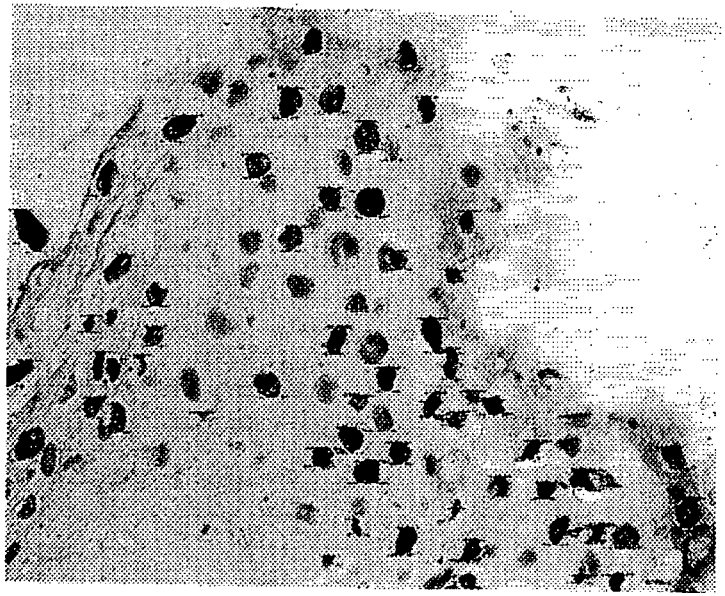


Figure 9. Mild cervical dysplasia showing intranuclear localization of hPV antigens in cells near the surface. Note that hPV antigens are not only confined to koilocytotic cells (immunoperoxidase-papillomavirus antigens, no counterstain). From Kurman et al (1982) with kind permission of the editor of *International Journal of Gynecological Pathology*.

degree of nuclear atypia. PV structural antigens were localized in the superficial layers in a pattern similar to that found in the mild dysplasias.

Severe dysplasia and carcinoma in situ. In the rare instances when PV antigens were localized within severe dysplasia, the positive cells were located in differentiated cells immediately on the surface; PV antigens were never localized in the underlying undifferentiated proliferating cells. More often PV antigens were localized in areas of mild and moderate dysplasia adjacent to the high-grade lesions.

Squamous carcinoma. PV antigens have not been identified thus far in invasive squamous carcinoma.

Limitations of Methodology

A positive reaction with the PAP technique indicates the presence of PV structural antigens. Virus that does not express viral antigens, i.e. viral DNA only, is not detected. In a preliminary study comparing the results of immunocytochemistry with molecular hybridization, over 90 per cent of cases of cervical dysplasia (mild, moderate and severe) were positive for PV antigens and/or PV DNA, but only 50 per cent showed evidence of PV antigens (Lancaster et al, in press).

The sensitivity of the PAP technique for the detection of PV antigens has not been determined, but correlation with transmission electron microscopy for the presence of viral particles indicates that immunocytochemistry detects twice as many positive cases as does electron microscopy (Ferenczy, Braun and Shah, 1981). Every specimen shown to contain viral particles by electron microscopy has been shown to be positive for PV antigens with immunocytochemistry. In contrast, cases positive by immunocytochemistry could take multiple attempts before viral particles were identified by electron microscopy (Jenson et al, 1982). The superiority of immunocytochemistry for the detection of PV in tissue sections is due to the fact that larger samples can be tested and that PV may be only focally distributed in a particular lesion (Kurman, Jenson and Lancaster, 1983).

Interpretation of Results

Comparison of the frequency of the morphological features of PV infection with the presence of PV-antigens revealed that the morphological changes were found approximately three times more frequently. The reasons for this discrepancy may be related to the limitations of the immunocytochemical method or to the biology of the virus. Periodic expression of the virus genome, as has been shown in juvenile laryngeal papillomas (Lack et al, 1980), could result in suspension of virus protein synthesis in cells that still show morphological manifestations of PV infection. Also, failure to detect PV antigens in the high-grade lesions may reflect a disturbance in virus assembly associated with neoplastic transformation, as occurs in animals. Intact virions are readily detectable in papillomas of wild cottontail rabbits and in bovine alimentary tract papillomas but cannot be detected in the carcinomas which arise in the benign lesions (Lancaster and Olson, 1982).

The immunological evidence therefore demonstrates for the first time that a venereally transmitted agent, associated with a distinctive benign lesion, can be linked through a series of morphological transitions to a neoplasm. The morphological transitions are subtle, and at present it is not always possible to distinguish a benign lesion with superimposed virus-induced atypia and associated hyperplasia from one that is neoplastic. Furthermore, whether all lesions recognized as being viral in origin are benign or whether a subset may progress to neoplasia is unknown at present. The immunocytochemical findings will undoubtedly lead to a re-evaluation of our concepts of cervical neoplasia and to modifications in the nomenclature of these lesions.

SUMMARY

The immunoperoxidase technique is relatively simple and inexpensive, and once established it can be easily adapted to any number of antigens. The method has already been employed to localize a wide variety of enzymes, proteins, polypeptide hormones, steroid hormones, immunoglobulins, and viral and protozoal antigens. The only limitations of the immunoperoxidase technique are the availability of specific antisera and the ability of the cellular

product being tested to retain its antigenicity through the process of fixation, dehydration and embedding. It is now apparent that, with the exception of a few labile antigens, most cellular products survive routine fixation to the extent that sufficient antigenic determinants remain to permit their recognition. Meaningful interpretation of the results, however, depends on thoughtful evaluation of the methodology. This requires careful immunological and tissue controls. Standardization of antibodies used as reagents, particularly in the rapidly burgeoning field of monoclonal antibodies, is essential in order that studies performed by different laboratories can be compared.

The application of immunocytochemistry to the study of gynaecological disease has been relatively recent, but it is apparent that this technique is a powerful tool with which to confirm and extend the morphological observations made over the last century. By taking advantage of the high degree of specificity of antibodies, immunocytochemistry has assumed a prominent role as a highly specific 'special stain'. Of perhaps even greater significance is the use of this technique to explore and characterize the biochemical features of cells within the framework of conventional morphology. Thus, gynaecological pathology is on the threshold of a new era in which the pathologist can now study the cellular manifestations of disease on both a functional and a morphological basis.

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